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# Familial multinodular goiter and Sertoli-Leydig cell tumors associated with a large intragenic in-frame *DICER1* deletion

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## Abstract

**Objective.** Familial multinodular goiter (MNG), with or without ovarian Sertoli-Leydig cell tumor (SLCT), has been linked to *DICER1* syndrome. We aimed to search for the presence of a germline *DICER1* mutation in a large family with a remarkable history of MNG and SLCT, and to further explore the relevance of the identified mutation.

**Design and methods.** Sanger sequencing, Fluidigm Access Array and multiplex ligation-dependent probe amplification (MLPA) techniques were used to screen for *DICER1* mutations in germline DNA from 16 family members. Where available, tumor DNA was also studied. mRNA and protein extracted from carriers' lymphocytes were used to characterize the expression of the mutant *DICER1*.

**Results.** Nine of 16 tested individuals carried a germline, in-frame *DICER1* deletion (c.4207-41\_5364+1034del), which resulted in the loss of exons 23 and 24 from the cDNA. The mutant transcript does not undergo nonsense-mediated decay and the protein is devoid of specific metal ion-binding amino acids (p.E1705 and p.D1709) in the RNase IIIb domain. In addition, characteristic somatic 'second hit' mutations in this region were found on the other allele in tumors.

**Conclusions.** Patients with *DICER1* syndrome usually present a combination of a typically truncating germline *DICER1* mutation and a tumor-specific hotspot missense mutation within the sequence encoding the RNase IIIb domain. The in-frame deletion found in this family suggests that the germline absence of p.E1705 and p.D1709, which are crucial for RNase IIIb activity, may be enough to permit *DICER1* syndrome to occur.

## Introduction

Multinodular goiter (MNG) is a common disorder of the thyroid gland, characterized by thyroid enlargement due to the development of multiple hyperplastic nodules (1, 2). As opposed to toxic MNG, the non-toxic subtype does not result from hyperthyroidism, hypothyroidism or inflammation. The exact causes of MNG are not well understood. Iodine deficiency has been established as the main cause of endemic goiter (2, 3), whereas genetic defects may play a major role in the pathogenesis of sporadic and familial non-toxic MNG. Two loci have been linked to familial MNG; *MNG1* on chromosome 14q (4) and *MNG2* on the X chromosome (5). In 2011, Rio Frio and coworkers (6) identified germline mutations in *DICER1* (on chromosome 14q32) as the cause of familial MNG with or without ovarian Sertoli–Leydig cell tumor (SLCT) (7), adding a new phenotype to the *DICER1* syndrome (OMIM #601200). Since then, several articles have reported patients with germline *DICER1* mutations who have MNG alone or in combination with other known *DICER1*-related diseases (8, 9, 10).

*DICER1* syndrome, first identified by Hill and colleagues in 2009 (11), is a rare pediatric tumor predisposition syndrome caused by germline typically truncating mutations in *DICER1*. A spectrum of rare, mostly pediatric-onset tumors characterizes the syndrome, including pleuropulmonary blastoma, SLCT, pediatric cystic nephroma, MNG, thyroid carcinoma, and other rare sarcomas and dysplasias (7). Somatic missense mutations affecting the nucleotides encoding the critical metal ion-binding residues within the catalytic RNase IIIb domain are found in the tumors (7). In the case of MNG, it has also been demonstrated that individual nodules may harbor distinct somatic RNase IIIb hotspot mutations (12, 13).

Here, we aimed to investigate this large family with a remarkable history of MNG and SLCT for presence of germline and somatic *DICER1* mutations and to further explore the relevance of the identified mutations.

## Subjects and methods

### Subjects

The studied pedigree comprised 16 individuals from a family of Italian descent. All individuals or their parents gave written informed consent. The study was approved by the Institutional Review Board of the Faculty of Medicine of McGill University no. A12-M117-11A. All procedures were performed in accordance with the ethical standards of the Helsinki Declaration.

### *DICER1* genetic testing

Blood samples were obtained from all subjects and 7 formalin-fixed, paraffin-embedded (FFPE) tumor blocks were available from 6 individuals ( $n = 5$  MNG, 1 SLCT, and 1 breast fibroadenoma). Genomic DNA was extracted from peripheral blood samples using the Gentra Puregene Blood Kit (QIAGEN) and from FFPE tumor tissue using the QIAamp DNA FFPE Tissue Kit (QIAGEN) according to the manufacturer's instructions.

Germline DNA from two patients was sequenced using a custom Fluidigm Access Array (14), which selectively targets the exons, exon–intron boundaries and 3'UTR of *DICER1*. In addition, multiplex ligation-based probe amplification (MLPA) assay was performed to screen for *DICER1* deletions or duplications (15). Long-range PCR was performed using TaKaRa LA Taq DNA Polymerase with GC Buffer (TaKaRa) to determine the deletion breakpoints. Primers were designed to amplify a 4801 bp DNA fragment of *DICER1* from intron 22 to intron 24. PCR products were gel extracted using QIAquick Gel Extraction Kit (QIAGEN) and then, Sanger sequenced.

The regions encoding the *DICER1* RNase IIIa and IIIb domains were PCR amplified and Sanger sequenced in DNA extracted from the lesions to screen for hotspot mutations.

### **RNA analysis and cloning assay**

Lymphoblastoid cell lines (LCLs) were established from peripheral blood lymphocytes of two affected individuals. LCLs from healthy, *DICER1* wild-type individuals were used as controls. Total RNA was extracted from LCLs using TRIzol reagent (Thermo Fisher Scientific). cDNA was synthesized using SuperScript III First-Strand Synthesis System (Thermo Fisher Scientific) and was PCR-amplified using Platinum Pfx DNA Polymerase (Thermo Fisher Scientific). Primers were designed to amplify a 1521 bp cDNA fragment from the end of exon 21 to the end of exon 25. PCR products were cloned into pCR-Blunt II-TOPO vector using Zero Blunt TOPO PCR Cloning Kit (Life Technologies). One Shot chemically competent *E. coli* cells were transformed with the pCR-Blunt II-TOPO construct. After selection with kanamycin, the constructs were Sanger sequenced using M13 primers.

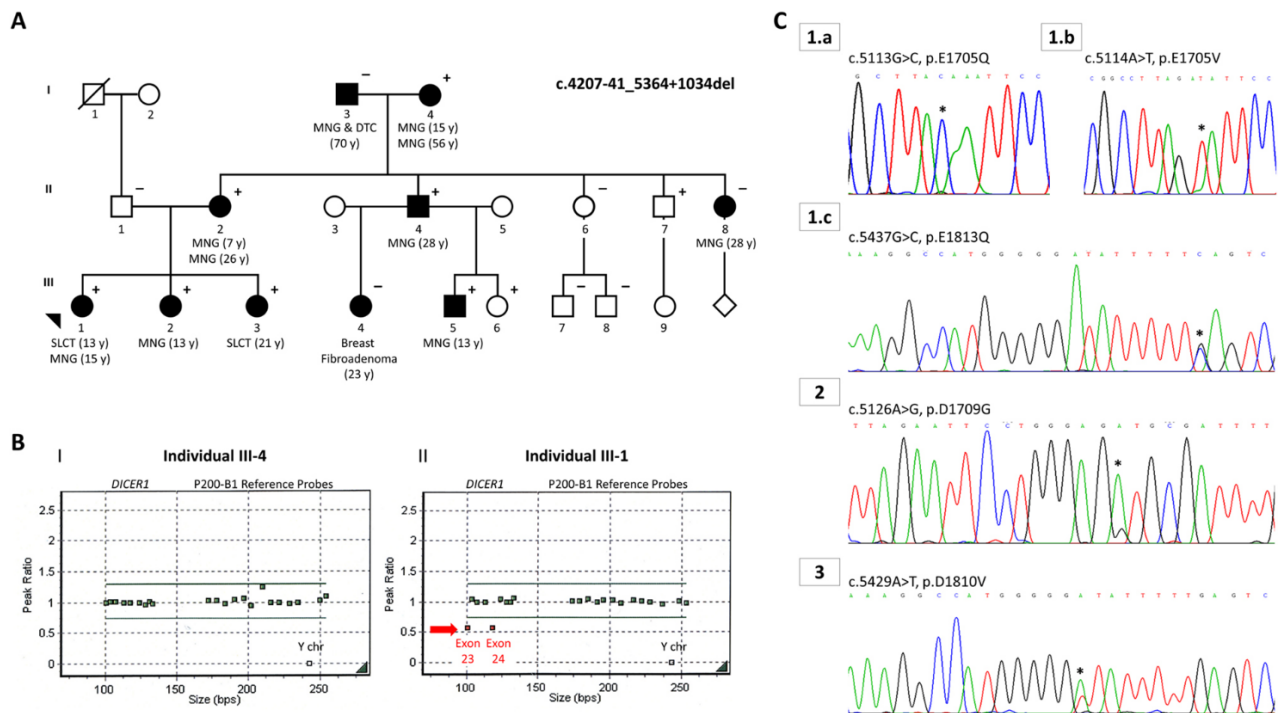
### **DICER1 immunodetection**

Proteins were extracted from LCLs using RIPA lysis buffer (10 mM Tris-HCl pH 7.3, 10% sodium deoxycholate, 10% Triton X-100, 150 mM NaCl, 1 mM EDTA, 50 mM NaF, 10 mM beta-glycerophosphate) supplemented with Complete, Mini, EDTA-free protease inhibitor cocktail (Roche). Western blot was performed using standard procedures and rabbit polyclonal anti-DICER1 antibody (1:2000, A301-936A, Bethyl Laboratories, Inc., Montgomery, TX, USA) or mouse monoclonal anti- $\beta$ -tubulin antibody, clone AA2 (1:1000, 05-661, Millipore Sigma).

## **Results**

### **Clinical information**

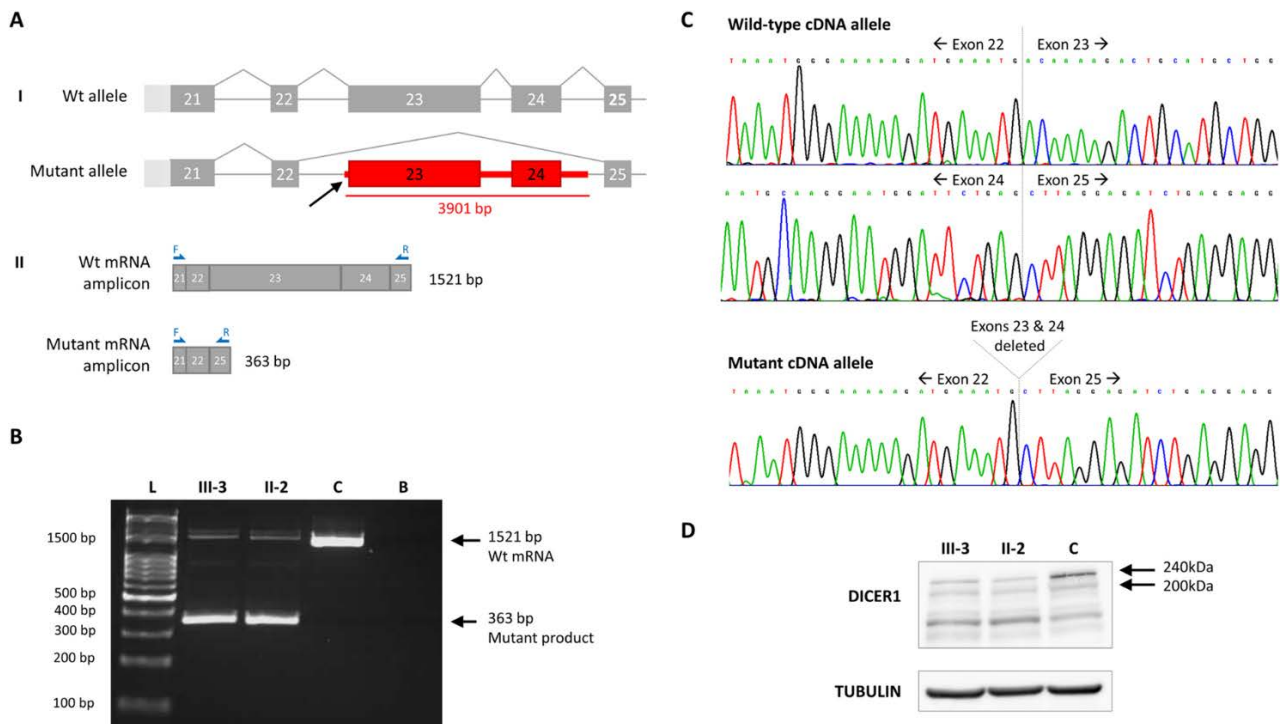
At age 13 years, the proband (individual III-1 in pedigree, Fig. 1A) underwent right unilateral oophorectomy to remove an SLCT, and two years later, a total thyroidectomy was performed due to symptomatic non-toxic MNG. The elder of her two sisters underwent left unilateral oophorectomy at age 21 years due to an SLCT, and the other sister had a total thyroidectomy at age 13 years due to MNG (individuals III-3 and III-2, respectively). Several maternal relatives had a history of MNG. In addition, individual III-4 (proband's cousin) had breast fibroadenoma and individual I-3 (proband's grandfather) had MNG with a focus of differentiated thyroid cancer (Fig. 1A). Given that young-onset MNG, alone or in combination with SLCT, is highly suggestive of *DICER1* syndrome, genetic testing was undertaken.



**Figure 1.** *DICER1* screening results. (A) Family pedigree: carriers of the germline *DICER1* in-frame deletion are indicated with ‘+’ and non-carriers with ‘-’. The arrowhead indicates the proband. SLCT, Sertoli-Leydig cell tumor; MNG, multinodular goiter; DTC, differentiated thyroid carcinoma. (B) Representation of MLPA results for non carrier (individual III-4) and a deletion carrier (III-1). A probe mix for *DICER1* is used in combination with the P200-B1 set of control and reference probes from MRC-Holland (Amsterdam, The Netherlands). Both individuals are females; hence, the peak ratio value of 0 for the Y chromosome. No deletions or duplications in *DICER1* were found in the non-carrier, with all ratios for *DICER1* exons (green squares, left) falling within the normal peak ratio range of 0.7–1.3 (green horizontal lines). Lower peak ratio values representing copy number loss were obtained for exons 23 and 24 in the deletion carriers (red arrow). Graphics generated with GeneMarker, v.1.70 (<http://www.softgenetics.com/GeneMarker.html>). (C) Chromatograms showing the somatic hotspot mutations found in tumors from *DICER1* deletion carriers. Panel 1 shows the mutations identified in the proband’s (individual III-1) tumors. 1.a and 1.b show mutations found in different nodules from the MNG. 1.c shows the mutation found in the SLCT. Panel 2 shows the mutation in the MNG of the proband’s grandmother (individual I-4). Panel 3, mutation found in the MNG of the proband’s sister (individual III-2). Mutations are indicated by an asterisk.

## Germline and somatic mutation analyses

Full sequencing of *DICER1* in the proband’s germline DNA did not identify any point mutations or small insertions/deletions. However, MLPA showed copy loss for exons 23 and 24, corresponding to a deletion (Fig. 1B). Analysis of the DNA breakpoints identified a heterozygous germline in-frame *DICER1* deletion spanning 3901 bases (c.4207-41\_5364+1034del) (Supplementary Fig. 1, see section on supplementary data given at the end of this article). It comprised 41 bases of the 3’ end of intron 22, exon 23, intron 23, exon 24 and 1034 bases of intron 24 (Fig. 2A panel I and Supplementary Fig. 1).



**Figure 2.** Effect of *DICER1* in-frame deletion on mRNA and at protein level. (A) Graphic representation of wild-type and mutant *DICER1* DNA alleles (Panel I), and the corresponding spliced mRNA transcripts (Panel II). In Panel I, boxes represent exons, horizontal lines represent introns, red shading indicates the deleted region and the arrow indicates the first deleted base. (B) 1.5% agarose gel showing the results of PCR amplification of a cDNA fragment spanning from exon 21 to exon 25 (1521 bp). Given that the DNA deletion begins in intron 22 and ends in intron 24, following mRNA splicing, a product of 363 bp comprising exon 22 spliced to exon 25, and lacking exons 23 and 24, may be expected. This 363 bp product from the mutant allele, in addition to the 1521 bp product from the wild-type allele, were detected in mRNA from LCLs from individual III-3 (proband's sister) and individual II-2 (proband's mother). Only the wild-type fragment was detected in a control sample (C). 'L' is the 100 bp DNA Ladder H3 RTU (GeneDireX, Ontario, Canada), and 'B' is a blank control PCR reaction. (C) Sequencing of PCR products obtained in B, showed a loss of exon 23 and 24 in the mutation carriers. (D) Western blot showing DICER1 and tubulin protein expression in carriers (individuals III-3 and II-2) and in a control (C). Wt, wild type.

Testing of a further fifteen family members revealed eight additional carriers of the in-frame deletion, including the proband's two sisters (individuals III-2 and III-3) and her mother (II-2) (Fig. 1A). All carriers had been diagnosed with MNG or SLCT (or both in the case of the proband), except for two individuals who were healthy as of September 2017 at ages 42 and 10 years (individuals II-7 and III-6, respectively). Two non-carriers had MNG (individuals I-3 and II-8, diagnosed at age 70 and 28 years respectively), one with a focus of differentiated thyroid cancer (DTC), papillary subtype. An additional female non-carrier was diagnosed with a breast fibroadenoma at 22 years of age (individual III-4).

Screening for somatic *DICER1* hotspot mutations was performed on 7 tumor samples from 6 individuals. Two individual nodules from the proband's MNG each harbored a distinct RNase IIIb hotspot mutation (c.5113G>C, p.E1705Q and c.5114A>T, p.E1705V; Fig. 1C, chromatograms 1.a

and 1.b), and a c.5437G>C, p.E1813Q mutation was found in her SLCT (Fig. 1C, chromatogram 1.c). Two MNG samples from deletion carriers also had somatic RNase IIIb hotspot mutations (individual I-4 had c.5126A>G, p.D1709G, Fig. 1C, chromatogram 2; and individual III-2 had c.5429A>T, p.D1810V; Fig. 1C, chromatogram 3), although we did not find a somatic hotspot mutation in the MNG from individual II-2. The grandfather's MNG (individual I-3) and the cousin's breast fibroadenoma (individual III-4) did not have mutations in the sequence encoding the RNase IIIa or IIIb domains of DICER1 (data not shown). The latter two individuals were non-carriers of the familial mutation.

### Expression of the germline in-frame deletion transcript/protein

PCR amplification of a region from exon 21 to exon 25 was performed on cDNA synthesized from germline RNA from control patients and deletion carriers. The full amplified region spanned 1521 base pairs (Fig. 2A panel II). A band corresponding to a wild-type (wt) mRNA transcript (1521 bp) was present in control samples. However, in *DICER1* deletion carriers, we identified two bands: one corresponding to the wt mRNA transcript and another corresponding to the mutant mRNA transcript (363 bp) (Fig. 2A and B). As the LCLs used for mRNA extraction were not treated with an inhibitor of nonsense-mediated decay (NMD), the PCR results indicate that the mutant transcript was not subjected to NMD (Fig. 2B). Sequencing of the PCR products confirmed that the mutant mRNA band (363 bp) lacked exons 23 and 24 (Fig. 2C). Carriers expressed a lower level of wt DICER1 protein compared to controls, as shown by Western blot (Fig. 2D). The mutant transcript is predicted to be translated into a shorter DICER1 protein of ~200 kDa, devoid of 107 of 165 amino acids constituting the RNase IIIb domain. However, this could not be proven as a protein of ~200 kDa was also observed in the control sample (Fig. 2D), possibly a naturally occurring alternate protein isoform.

## Discussion

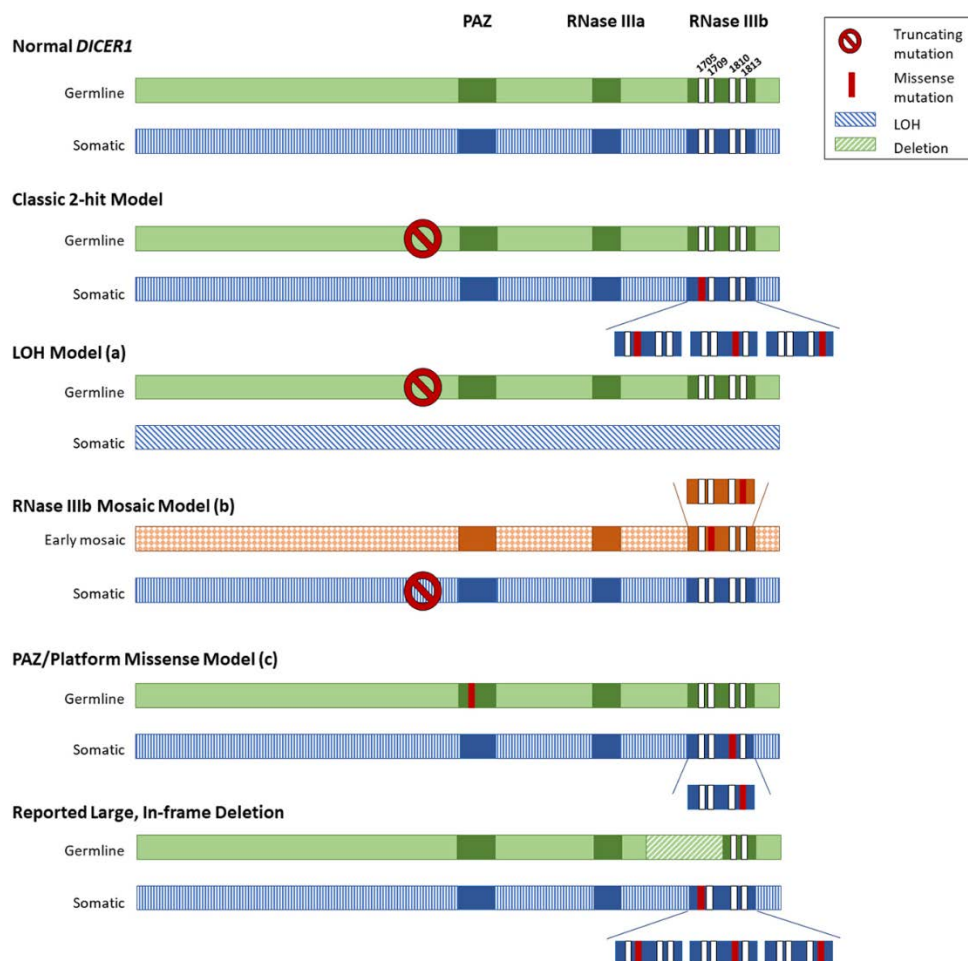
Hyperplastic thyroid abnormalities are a common finding in patients with germline *DICER1* mutations (7), and risk of thyroid carcinoma in *DICER1* syndrome patients is elevated (13, 16). The penetrance of heterozygous germline *DICER1* pathogenic variants for neoplasia is seemingly low (<15%), although for some conditions such as MNG, it may be higher (6, 17, 18). Indeed, in a recent study by Khan and coworkers, by age 40 years, the cumulative incidence of MNG or thyroidectomy was observed to be 75% in women and 17% in men with *DICER1* syndrome, compared to 8% and 0% of control women and men, respectively (13).

In this study in which 8 relatives had MNG, we screened for *DICER1* mutations using MLPA and unveiled a germline in-frame *DICER1* deletion in nine family members. This emphasizes the effectiveness of MLPA in detecting single- or multiple-exon insertions and deletions, which would be missed by Sanger sequencing (15) and might also be overlooked by targeted, massively parallel, panel-based gene sequencing approaches.

In our family, the deletion in *DICER1* appears to be highly penetrant as seven of nine carriers (77%) had MNG, SLCT or both diseases. The remaining two carriers have no history of syndromic disease as of September 2017. Typical somatic hotspot mutations were found in three out of four carriers for whom tumor was available. The absence of a second hit in individual II-2 could be due to a lack of sensitivity of Sanger sequencing in detecting a low frequency variant. However, it is possible that individual II-2 does not have a somatic *DICER1* mutation, as seen in thyroid nodules from some germline *DICER1* mutation carriers (13). The two non-carriers with thyroid conditions (MNG and/or thyroid cancer) may suggest the involvement of different genetic or environmental factors. In fact, the region of Italy from which the family originates and resides has a high prevalence of MNG (19, 20).



Studies of *DICER1*-associated tumors have revealed biallelic *DICER1* mutations. The two-hit genetic model in *DICER1* syndrome involves one germline typically truncating mutation (a nonsense or a frame-shift mutation) and a somatic missense mutation in the region encoding the RNase IIIb domain (Fig. 3). Germline mutations occur across the entire gene, whereas somatic mutations appear to be clustered at bases encoding the metal ion-binding or adjacent residues (p.E1705, p.D1709, p.D1810 and p.E1813) of RNase IIIb domain, so-called ‘hotspots’ (7, 12, 21). This domain is crucial for the production of mature microRNAs from the 5p arm of precursor microRNAs (22). Amino acid substitutions at these hotspots interfere with the catalytic site of *DICER1* leading to a 3p mature microRNA strand bias (22), which results in altered microRNA-mediated posttranscriptional gene regulation (23).



**Figure 3.** Genetic models in *DICER1* syndrome. Representations of the different genetic models found in patients with *DICER1* syndrome in comparison to a healthy individual with two wild-type copies of *DICER1*. From top to bottom, we show a normal *DICER1* genotype, the classic 2-hit model of *DICER1* syndrome, the loss-of-heterozygosity model, the RNase IIIb mosaic model, PAZ/Platform missense model and the in-frame deletion described in this study. Relevant domains are highlighted in dark colors. Germline is shown in solid green, early mosaic in orange diamonds and somatic with blue stripes. White rectangles depict the four key amino acids (‘hotspots’) in RNase IIIb domain.



Variations of this model have also been described (Fig. 3): (a) Germline truncating mutation accompanied by somatic loss of heterozygosity (LOH) has been frequently observed in pineoblastoma, (24) and has also been seen in a Wilms tumor (25) and a pituitary blastoma (14); (b) somatic mosaicism for an RNase IIIb hotspot mutation. In this case, the cells that give rise to a tumor acquire a truncating mutation as the second hit (26, 27). This scenario is associated with an early-onset and severe multifocal phenotype compared to patients with the classical two-hit model (26); and (c) germline missense mutations in the region coding for the PAZ/platform domain accompanied by a somatic hotspot mutation (6, 28). Here, we suggest another model, wherein heterozygous loss of two relevant metal ion-binding residues (i.e. p.E1705 and p.D1709) in the germline appears to be sufficient to provoke DICER1 syndrome (Fig. 3).

Five in-frame deletions in *DICER1* have been previously published. Rio Frio and coworkers investigated two families with history of MNG, which resulted from in-frame deletions (6). One family was found to bear a missense mutation that created a *de novo* splice site, producing a transcript with an in-frame deletion of the first 21 base pairs of exon 16 (r.2437\_2457del21, p.I813\_Y819del). The other family had a splice site mutation (c.2805-1G>T) that resulted in an in-frame deletion of exon 18 (6). Both mutations altered the structure of DICER1 by eliminating part of the Platform or PAZ domains (Fig. 3). A similar case was presented by Yoshida and colleagues. They described a 12-year-old girl diagnosed with anaplastic sarcoma of the kidney and follicular thyroid carcinoma who had a germline insertion/deletion in *DICER1* (c.5426\_5442delGGGATATTTTGTAGTC GlnsCA, p.G1809\_S1814delinsA). Although a small deletion, it resulted in DICER1 devoid of three key residues (p.G1809, p.D1810 and p.E1813) in the RNase IIIb domain (29). In the study by Rath and coworkers (10), they found a germline in-frame deletion (c.5221\_5232delAACAACACCATC, p.N1741\_I1744del) in a 9-year-old boy with cystic nephroma, MNG and pleuropulmonary blastoma (PPB). The father, who had been diagnosed with PPB and MNG, was also a carrier. Interestingly, the elimination of four amino acids in RNase IIIb domain, although not hotspot residues, was able to predispose to DICER1 syndrome. Finally, Doros and coworkers described a child with abdominal embryonal rhabdomyosarcoma bearing c.4259\_4261delGAG (p.1418\_1420delE). Based on the amino acid conservation and the location of the glutamic acid in an acidic portion of DICER1, they proposed that the change would be pathogenic (30) although no functional evidence was provided. In addition to these published cases, ClinVar lists two other in-frame deletions, c.3516\_3525delTACAGCAATTinsA (p.T1173\_I1175del), which is reported to be pathogenic by a single submitter, but with no supporting details (<https://www.ncbi.nlm.nih.gov/clinvar/variation/429158/>), and c.4260\_4262delGGA (p.E1420del), which has the same end-result at the protein level as the in-frame deletion described by Doros and coworkers (30), but in this case, it is reported to be likely benign by multiple submitters (<https://www.ncbi.nlm.nih.gov/clinvar/variation/133971/>). The in-frame deletion shown in our study, which spanned 3901 bases, is the largest intragenic *DICER1* deletion described in the literature.

In the thyroid gland, DICER1 and microRNAs are necessary for accurately establishing thyroid follicles and hormone synthesis. Normal thyroid highly expresses let-7 isoforms, miR-15/miR-16 cluster and other miRNAs (31). Conversely, these microRNAs have been found to be downregulated in thyroid tumors (32). Using mouse models, the specific depletion of *DICER* in thyrocytes led to loss of follicular architecture, was associated with functional aberrations, caused downregulation of thyroid cell differentiation markers (33) and also led to induction of cell proliferation (34). Moreover, several microRNAs predicted to target the 3'-UTR of thyroid differentiation genes (e.g. *NIS*, *TG* or *DUOX1*) and thyroid transcription factors (e.g. PAX8, NKX2-1 and FOXE1) were found to be downregulated in *Dicer1* knockout models (35).

The development of MNG has been described to start with a goitrogenic stimulus such as an environmental factor (i.e. iodine deficiency) or a genetic alteration that causes diffuse thyroid hyperplasia. Then, an increase in the mutagenic load of the thyroid occurs either consequent to an increase in the H<sub>2</sub>O<sub>2</sub> production and free radical formation due to thyroid hormone synthesis, or by a

failure of mutation repair due to higher thyroid cell replication rate. Finally, some of the resulting cell clones will contain an advantageous mutation that causes growth of multiple thyroid nodules (2).

In agreement with this, de Kock and coworkers proposed a model of *DICER1*-related MNG development in which a germline loss-of-function *DICER1* mutation creates a haploinsufficient environment leading to diffuse thyroid hyperplasia. Subsequently, the acquisition of an RNase IIIb mutation on the other allele results in the formation of a monoclonal nodule. Over time, more nodules appear as more RNase IIIb mutations occur in thyroid cells, ultimately resulting in MNG (12).

In this study, we hypothesize that the loss of crucial amino acids in RNase IIIb due to the germline *DICER1* in-frame deletion leads to a decreased production of 5p microRNAs, which result in thyroid cell proliferation. It has been shown that let-7a and miR-345 (both 5p microRNAs) are downregulated in *DICER1*-related goiter, compared to normal thyroid tissue (6). Moreover, low levels of let-7 and miR-345 have been recurrently found in thyroid tumors (32, 36). After the induction of hyperplasia, *DICER1* ‘hotspot’ mutations in RNase IIIb may occur on the other allele (as observed in the proband, individual I-4 and individual III-2), giving rise to cell clones that would develop into thyroid nodules.

Taken together, these results suggest that loss of one or more clusters of key residues (p.E1705 and p.D1709 and/or p.G1809, p.D1810 and p.E1813) in the domain involved in premature microRNA cleavage is enough to ‘lay the groundwork’ for *DICER1* syndrome to occur, since in nearly all cases, the tumors occurring in *DICER1* syndrome require a second hit in exons encoding the RNase IIIb domain, and conversely, nearly all of these somatic RNase IIIb hits occur in the context of an inactivating mutation on the other allele. In this study, we found a novel in-frame *DICER1* deletion, which resulted in a mutant transcript lacking exons 23 and 24. The resulting protein, if expressed in its entirety, would lack two metal ion-binding amino acids in the RNase IIIb domain, which are crucial for *DICER1* catalytic activity. Therefore, in-frame germline mutations must be carefully analyzed when assessing their potential to cause *DICER1* syndrome as they may eliminate bases encoding for physiologically critical amino acids.

## Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/EJE-17-0904>.

## Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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## References

1. Prades JM, Dumollard JM, Timoshenko A, Chelikh L, Michel F, Estour B & Martin C. Multinodular goiter: surgical management and histopathological findings. *European Archives of Oto-Rhino-Laryngology* 2002 259 217–221. (<https://doi.org/10.1007/s00405-002-0455-0>)
2. Hegedüs L, Paschke R, Krohn K & Bonnema SJ. Multinodular goiter. In *Endocrinology: Adult and Pediatric*, 7th ed., chapter 90, pp 1570–1583. Eds JL Jameson & LJ De Groot. Philadelphia: Elsevier, 2016.
3. Pinchera A, Aghini-Lombardi F, Antonangeli L & Vitti P. Multinodular goiter. Epidemiology and prevention. *Annali Italiani di Chirurgia* 1996 67 317–325.
4. Bignell GR, Canzian F, Shayeghi M, Stark M, Shugart YY, Biggs P, Mangion J, Hamoudi R, Rosenblatt J, Buu P et al. Familial nontoxic multinodular thyroid goiter locus maps to chromosome 14q but does not account for familial nonmedullary thyroid cancer. *American Journal of Human Genetics* 1997 61 1123–1130. (<https://doi.org/10.1086/301610>)
5. Capon F, Tacconelli A, Giardina E, Sciacchitano S, Bruno R, Tassi V, Trischitta V, Filetti S, Dallapiccola B & Novelli G. Mapping a dominant form of multinodular goiter to chromosome Xp22. *American Journal of Human Genetics* 2000 67 1004–1007. (<https://doi.org/10.1086/303095>)
6. Rio Frio T, Bahubeshi A, Kanellopoulou C, Hamel N, Niedziela M, Sabbaghian N, Pouchet C, Gilbert L, O'Brien PK, Serfas K et al. DICER1 mutations in familial multinodular goiter with and without ovarian Sertoli-Leydig cell tumors. *JAMA* 2011 305 68–77. (<https://doi.org/10.1001/jama.2010.1910>)
7. Foulkes WD, Priest JR & Duchaine TF. DICER1: mutations, microRNAs and mechanisms. *Nature Reviews Cancer* 2014 14 662–672. (<https://doi.org/10.1038/nrc3802>)
8. Darrat I, Bedoyan JK, Chen M, Schuette JL & Lesperance MM. Novel DICER1 mutation as cause of multinodular goiter in children. *Head and Neck* 2013 35 E369–E371. (<https://doi.org/10.1002/hed.23250>)
9. Rossing M, Gerdes AM, Juul A, Rechnitzer C, Rudnicki M, Nielsen FC & Vo Hansen T. A novel DICER1 mutation identified in a female with ovarian Sertoli-Leydig cell tumor and multinodular goiter: a case report. *Journal of Medical Case Reports* 2014 8 112. (<https://doi.org/10.1186/1752-1947-8-112>)
10. Rath SR, Bartley A, Charles A, Powers N, Baynam G, Jones T, Priest JR, Foulkes WD & Choong CS. Multinodular Goiter in children: an important pointer to a germline DICER1 mutation. *Journal of Clinical Endocrinology and Metabolism* 2014 99 1947–1948. (<https://doi.org/10.1210/jc.2013-3932>)
11. Hill DA, Ivanovich J, Priest JR, Gurnett CA, Dehner LP, Desruisseau D, Jarzembowski JA, Wikenheiser-Brokamp KA, Suarez BK, Whelan AJ et al. DICER1 mutations in familial pleuropulmonary blastoma. *Science* 2009 325 965. (<https://doi.org/10.1126/science.1174334>)

12. de Kock L, Bah I, Revil T, Berube P, Wu MK, Sabbaghian N, Priest JR, Ragoussis J & Foulkes WD. Deep sequencing reveals spatially distributed distinct hot spot mutations in DICER1-related multinodular goiter. *Journal of Clinical Endocrinology and Metabolism* 2016 101 3637–3645. (<https://doi.org/10.1210/jc.2016-1328>)
13. Khan NE, Bauer AJ, Schultz KAP, Doros L, Decastro RM, Ling A, Lodish MB, Harney LA, Kase RG, Carr AG et al. Quantification of thyroid cancer and multinodular goiter risk in the DICER1 syndrome: a family-based cohort study. *Journal of Clinical Endocrinology and Metabolism* 2017 102 1614–1622. (<https://doi.org/10.1210/jc.2016-2954>)
14. de Kock L, Sabbaghian N, Plourde F, Srivastava A, Weber E, BouronDal Soglio D, Hamel N, Choi JH, Park SH, Deal CL et al. Pituitary blastoma: a pathognomonic feature of germ-line DICER1 mutations. *Acta Neuropathologica* 2014 128 111–122. (<https://doi.org/10.1007/s00401-014-1285-z>)
15. Sabbaghian N, Srivastava A, Hamel N, Plourde F, Gajtko-Metera M, Niedziela M & Foulkes WD. Germ-line deletion in DICER1 revealed by a novel MLPA assay using synthetic oligonucleotides. *European Journal of Human Genetics* 2014 22 564–567. (<https://doi.org/10.1038/ejhg.2013.215>)
16. de Kock L, Sabbaghian N, Soglio DB, Guillerman RP, Park BK, Chami R, Deal CL, Priest JR & Foulkes WD. Exploring the association between DICER1 mutations and differentiated thyroid carcinoma. *Journal of Clinical Endocrinology and Metabolism* 2014 99 E1072–E1077. (<https://doi.org/10.1210/jc.2013-4206>)
17. Doros L, Schultz KA, Stewart DR, Bauer AJ, Williams G, Rossi CT, Carr A, Yang J, Dehner LP, Messinger Y et al. DICER1-related disorders. In *GeneReviews(R)*. Eds RA Pagon, MP Adam, HH Ardinger, SE Wallace, A Amemiya, LJH Bean, TD Bird, N Ledbetter, HC Mefford, RJH Smith et al. Seattle (WA): University of Washington, Seattle; 2014.. Available at <https://www.ncbi.nlm.nih.gov/books/NBK196157/>
18. Rutter MM, Jha P, Schultz KA, Sheil A, Harris AK, Bauer AJ, Field AL, Geller J & Hill DA. DICER1 mutations and differentiated thyroid carcinoma: evidence of a direct association. *Journal of Clinical Endocrinology and Metabolism* 2016 101 1–5. (<https://doi.org/10.1210/jc.2015-2169>)
19. Aghini Lombardi F, Fiore E, Tonacchera M, Antonangeli L, Rago T, Frigeri M, Provenzale AM, Montanelli L, Grasso L, Pinchera A et al. The effect of voluntary iodine prophylaxis in a small rural community: the Pescopagano survey 15 years later. *Journal of Clinical Endocrinology and Metabolism* 2013 98 1031–1039. (<https://doi.org/10.1210/jc.2012-2960>)
20. Fiore E, Tonacchera M & Vitti P. Influence of iodization programmes on the epidemiology of nodular goitre. *Best Practice and Research: Clinical Endocrinology and Metabolism* 2014 28 577–588. (<https://doi.org/10.1016/j.beem.2014.04.002>)
21. Heravi-Moussavi A, Anglesio MS, Cheng SW, Senz J, Yang W, Prentice L, Fejes AP, Chow C, Tone A, Kalloger SE et al. Recurrent somatic DICER1 mutations in nonepithelial ovarian cancers. *New England Journal of Medicine* 2012 366 234–242. (<https://doi.org/10.1056/NEJMoa1102903>)
22. Anglesio MS, Wang Y, Yang W, Senz J, Wan A, Heravi-Moussavi A, Salamanca C, Maines-Bandiera S, Huntsman DG & Morin GB. Cancer-associated somatic DICER1 hotspot mutations cause defective miRNA processing and reverse-strand expression bias to

- predominantly mature 3p strands through loss of 5p strand cleavage. *Journal of Pathology* 2013 229 400–409. (<https://doi.org/10.1002/path.4135>)
23. Bahubeshi A, Tischkowitz M & Foulkes WD. miRNA processing and human cancer: DICER1 cuts the mustard. *Science Translational Medicine* 2011 3 111ps146. (<https://doi.org/10.1126/scitranslmed.3002493>)
  24. de Kock L, Sabbaghian N, Druker H, Weber E, Hamel N, Miller S, Choong CS, Gottardo NG, Kees UR, Rednam SP et al. Germ-line and somatic DICER1 mutations in pineoblastoma. *Acta Neuropathologica* 2014 128 583–595. (<https://doi.org/10.1007/s00401-014-1318-7>)
  25. Palculict TB, Ruteshouser EC, Fan Y, Wang W, Strong L & Huff V. Identification of germline DICER1 mutations and loss of heterozygosity in familial Wilms tumour. *Journal of Medical Genetics* 2016 53 385–388. (<https://doi.org/10.1136/jmedgenet-2015-103311>)
  26. Brenneman M, Field A, Yang J, Williams G, Doros L, Rossi C, Schultz KA, Rosenberg A, Ivanovich J, Turner J et al. Temporal order of RNase IIIb and loss-of-function mutations during development determines phenotype in DICER1 syndrome: a unique variant of the two-hit tumor suppression model. *F1000Research* 2015 4 214. (<https://doi.org/10.12688/f1000research.6746.1>)
  27. de Kock L, Wang YC, Revil T, Badescu D, Rivera B, Sabbaghian N, Wu M, Weber E, Sandoval C, Hopman SM et al. High-sensitivity sequencing reveals multi-organ somatic mosaicism causing DICER1 syndrome. *Journal of Medical Genetics* 2016 53 43–52. (<https://doi.org/10.1136/jmedgenet-2015-103428>)
  28. Chen J, Wang Y, McMonechy MK, Anglesio MS, Yang W, Senz J, Maines-Bandiera S, Rosner J, Trigo-Gonzalez G, Grace Cheng SW et al. Recurrent DICER1 hotspot mutations in endometrial tumours and their impact on microRNA biogenesis. *Journal of Pathology* 2015 237 215–225. (<https://doi.org/10.1002/path.4569>)
  29. Yoshida M, Hamanoue S, Seki M, Tanaka M, Yoshida K, Goto H, Ogawa S, Takita J & Tanaka Y. Metachronous anaplastic sarcoma of the kidney and thyroid follicular carcinoma as manifestations of DICER1 abnormalities. *Human Pathology* 2017 61 205–209. (<https://doi.org/10.1016/j.humpath.2016.06.024>)
  30. Doros L, Yang J, Dehner L, Rossi CT, Skiver K, Jarzembowski JA, Messinger Y, Schultz KA, Williams G, Andre N et al. DICER1 mutations in embryonal rhabdomyosarcomas from children with and without familial PPB-tumor predisposition syndrome. *Pediatric Blood Cancer* 2012 59 558–560. (<https://doi.org/10.1002/pbc.24020>)
  31. Marini F, Luzzi E & Brandi ML. MicroRNA role in thyroid cancer development. *Journal of Thyroid Research* 2011 2011 407123. (<https://doi.org/10.4061/2011/407123>)
  32. Pallante P, Battista S, Pierantoni GM & Fusco A. Deregulation of microRNA expression in thyroid neoplasias. *Nature Reviews Endocrinology* 2014 10 88–101. (<https://doi.org/10.1038/nrendo.2013.223>)
  33. Frezzetti D, Reale C, Cali G, Nitsch L, Fagman H, Nilsson O, Scarfo M, De Vita G & Di Lauro R. The microRNA-processing enzyme Dicer is essential for thyroid function. *PLoS ONE* 2011 6 e27648. (<https://doi.org/10.1371/journal.pone.0027648>)
  34. Rodriguez W, Jin L, Janssens V, Pierreux C, Hick AC, Urizar E & Costagliola S. Deletion of the RNaseIII enzyme dicer in thyroid follicular cells causes hypothyroidism with signs of

neoplastic alterations. PLoS ONE 2012 7 e29929. (<https://doi.org/10.1371/journal.pone.0029929>)

35. Fuziwara CS & Kimura ET. MicroRNAs in thyroid development, function and tumorigenesis. *Molecular and Cellular Endocrinology* 2017 456 44–50. (<https://doi.org/10.1016/j.mce.2016.12.017>)
36. Hu Y, Wang H, Chen E, Xu Z, Chen B & Lu G. Candidate microRNAs as biomarkers of thyroid carcinoma: a systematic review, metaanalysis, and experimental validation. *Cancer Medicine* 2016 5 2602–2614. (<https://doi.org/10.1002/cam4.811>)